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c-Rel Regulation of IL-2 Gene Expression May Be Mediated Through Activation of AP-1

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Summary

T cell activation by antigen/MHC induces the expression of several genes critical to the immune response, including interleukin-2. T cells from mice deficient for the NF-κB family member c-*rel* cannot activate IL-2 gene expression. However, mutating the NF-κB site in the IL-2 promoter has only moderate effects. To investigate additional ways c-Rel could regulate IL-2 gene expression, we determined whether c-rel overexpression could increase the activity of other transcription factors involved in IL-2 promoter regulation: NF-AT, Oct/OAP (ARRE-1), and AP-1. In Jurkat TAg cells, overexpression of c-Rel increased AP-1 activation ~17-fold. Moreover, AP-1 activity stimulated by anti-TCR Abs or PMA/ionomycin was further increased by c-Rel overexpression. c-Rel overexpression did not affect NF-AT or ARRE-1 activity. Additionally, overexpression of c-Rel activated the nonconsensus AP-1 site from the IL-2 promoter (NF-IL-2B), although to a lesser extent, approximately sixfold. AP-1 activation required both the DNA binding and transactivation domains of c-Rel. Our results may provide an explanation for the effect on IL-2 gene activation in c-*rel*-deficient mice.

The secretion of IL-2 by activated T cells is an important step in the initiation of an immune response. The primary regulation of IL-2 expression is at the level of transcription. The IL-2 promoter has been extensively characterized (for a review, see references 1–3), and contains binding sites for several transcription factors, including nuclear factor of activated T cells (NF-AT)[1], NF-κB, AP-1, and octamer-binding proteins. The exact nature of the interplay between these different transcription factors in the induction of IL-2 expression is unknown. Mutagenesis studies have demonstrated varying relative contributions of different sites within the IL-2 promoter (4). Mutating the AP-1 site (NF-IL-2B) decreased promoter expression to 11% of normal, while mutating the distal NF-AT site (NF-IL-2E) only decreased IL-2 promoter activity to 31% of normal. These suggested that more than one site contributes to IL-2 promoter activity. Consistent with this idea, if both the octamer sites are disrupted (NF-IL-2A, NF-IL-2D) or both the distal and proximal NF-AT sites are disrupted, IL-2 promoter activity is effectively abolished (5–7).

Interestingly, many of the transcription factors that regulate IL-2 gene expression appear to interact at multiple sites within the IL-2 promoter. For example, AP-1 family members can directly bind at the AP-1 site (NF-IL-2B) and are also capable of binding at the NF-AT (8, 9) and at ARRE-1 (10) sites in combination with NF-AT and octamer-binding proteins. However, mice deficient in c-*fos* (11), c-*jun* (12), *NF-κB p50* (3, 13), *Oct-2* (3, 14), or *NF-ATp* (15, 16) have no defects in IL-2 gene transcription. Of course, these transcription factors are members of large families; the knockouts examined may not include the critical family member required for IL-2 gene expression or functional redundancy within each family may compensate for the loss of one member. Consistent with this latter proposal, when AP-1 activity is disrupted, not by homologous recombination but by overexpression of a dominant negative c-jun, which can presumably block the activity of many AP-1 components (jun/fos family members), IL-2 gene expression is eliminated (17). Similarly, overexpression of a dominant negative $NF\text{-}AT_c$ can also block IL-2 gene induction (18). However, unlike knockouts of other transcription factors, a disruption of the c-Rel gene alone is sufficient to eliminate IL-2 gene expression (19).

c-Rel is a member of the larger NF-κB family, which includes p65 (RelA) and p50 (NF-κB1) (for reviews, see references 20–22). NF-κB family members all recognize a similar decameric motif (initially identified as the κB element in the κ-light chain gene intronic enhancer; 23), bind DNA as either homo- or heterodimers, and contain a simi-

[1]*Abbreviations used in this paper:* luc, luciferase; NF-AT, nuclear factor of activated T cells; Oct, Octamer; OAP, octamer-associated protein; CD28RE, CD28 response element; GST, glutathione-S transferase.

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lar structure within their DNA binding and dimerization domain, the *rel* homology domain. In the c-*rel*-deficient mice, although T cell development is normal, mature T cells fail to proliferate in response to mitogenic stimuli, such as Con A and anti-CD3/anti-CD28 (19). *c-rel*$^{-/-}$ T cells also fail to produce IL-2 upon stimulation with Con A or anti-CD3/anti-CD28, although other cytokines such as IL-5, TNF-α, and IFN-γ are affected to a lesser degree (24). The proliferative defect can be rescued by addition of exogenous IL-2. Therefore, it appears that one critical defect in c-*rel* deficient T cells is the block in IL-2 gene expression. However, the mode of c-Rel action on the IL-2 promoter is not clear, but it is unlikely to be mediated by the NF-κB site in the IL-2 promoter. Mutation of the NF-κB (NF-IL-2C) site had only a moderate effect on IL-2 promoter activity, to 20% of normal (1, 25). Additionally, the NF-κB family members that bind to this site in activated T cells have been shown to be either p50 homodimers (26) or p50/p65 heterodimers (27), and do not appear to contain c-Rel. Expression of both p50 and p65 is normal in c-*rel*-deficient mice. Therefore, it is unlikely that this NF-κB site in the IL-2 promoter mediates the defect observed in the c-*rel*-deficient mice. It is not clear whether the CD28RE may mediate the effect of c-Rel on IL-2 gene regulation; there is conflicting evidence on the role of NF-κB in binding and activating transcription from the CD28RE (reviewed in references 28, 29). Therefore, the loss of c-*rel* might affect other elements involved in IL-2 gene regulation.

NF-κB p65 overexpression has been shown to upregulate AP-1 activity (30), and it is possible that c-Rel has similar effects. We have examined the effect of c-Rel overexpression on NF-AT, AP-1, and ARRE-1 (Oct/OAP) activity. c-Rel dramatically increased activity from a consensus AP-1 reporter gene, ~17-fold. Additional increases were seen when c-Rel overexpression was combined with either anti-TCR or PMA/ionomycin stimulation. However, only minimal effects of c-Rel overexpression on NF-AT or ARRE-1 reporters were observed, even though both these elements contain AP-1 sites, albeit nonconsensus. When the isolated AP-1 site from either the distal NF-AT or ARRE-1 sites was examined, minimal effects of c-Rel overexpression was observed. It is probable that these sites cannot act independently and require cooperative binding of either NF-AT or octamer-binding factors. Overexpression of c-Rel also increased activity from a reporter containing the nonconsensus AP-1 site (NF-IL-2B) from the IL-2 promoter, although to a lesser degree than using a consensus AP-1 reporter, approximately sixfold. c-Rel activation of AP-1 required both its DNA binding and transcriptional activation domains. Our results may provide an explanation for the lack of IL-2 expression in c-*rel*-deficient mice.

Materials and Methods

Plasmids. The expression plasmids pCMV4, pCMV4 p65, pCMV4 c-rel 1–307, and pCMV4 c-rel 187–587 were previously described (31). Reporter constructs were generated by multimerizing oligos into the SalI site of the reporter pΔODLO (a gift from Dr. B. Starr and Dr. K. Yamamoto). All reporter constructs were sequenced to verify number and orientation of inserted oligos. 4×ARRE luc contains four copies of the ARRE-1 site from the IL-2 promoter in the reverse orientation using the following oligos:

5′-TCGAGTTTGAAAATATGTGTAATATGTAAAACATG-3′
3′-CAAACTTTTATACACATTATACATTTTGTACAGCT-5′

4×AP-1 luc contains four copies of the consensus AP-1 site from the metallothienein promoter in the reverse orientation using the following oligos:

5′-TCGAGTGACTCAGCGCG-3′
3′-CACTGAGTCGCGCAGCT-5′

3×NF-AT luc contains three copies of the distal NF-AT site from the IL-2 promoter in the forward orientation using the following oligos:

5′-TCGAGGGAGGAAAAACTGTTTCATACA-
GAAGGCGTG-3′
3′-CCCTCCTTTTTGACAAAGTATGTCTTCCG-
CACAGCT-5′

3×NF-κB luc contains three copies of the NF-κB site from the IL-2 receptor α promoter in the reverse orientation using the following oligos:

5′-TCGAGACGGCAGGGGAATCTCCCTCTCCG-3′
3′-CTGCCGTCCCCTTAGAGGGAGAGGCAGCT-5′

5×AP-1(NF-AT) contains five copies of the AP-1 site from the distal NF-AT site of the IL-2 promoter in the reverse orientation using the following oligos:

5′-TCGAGAACTGTTTCATACG-3′
3′-CTTGACAAAGTATGCAGCT-5′

4×AP-1(ARRE) contains four copies of the AP-1 site from the ARRE-1 site of the IL-2 promoter in the reverse orientation using the following oligos:

5′-TCGAGATATGTGTAATATG-3′
3′-CTATACACATTATACAGCT-5′

4×AP-1(NF-IL-2B) contains four copies of the AP-1/NF-IL-2B site from the IL-2 promoter, three in the reverse orientation, and one in the forward orientation, using the following oligos:

5′-TCGACAAAGAGTCATCAG-3′
3′-GTTTCTCAGTAGTCAGCT-5′

The site-directed mutants of c-rel, RxxRxR, and KKK were created using a two-step PCR-based mutagenesis using the proofreading Pwo polymerase (Boehringer Mannheim, Indianapolis, IN). These mutations recreate specific site-directed mutations that were originally generated in v-rel by Kumar et al. (32). The mutant RxxRxR changes R19 to A, R22 to A, and R24 to A. The mutant KKK changes K111 to N, K112 to Q, and K113 to Q. After PCR, the resulting mutants were sequenced to verify that the desired changes and no others had occurred. The oligos used to generate the mutations were as follows.
To generate RxxRxR:

5′-GGCGGGAATGGCTTTTGCATACAAATGTGAAGG-
GCG-3′
3′-TAACTTGTTGGGTCCGTCCGCCCTTACCGAAAA-
CGTA-5′

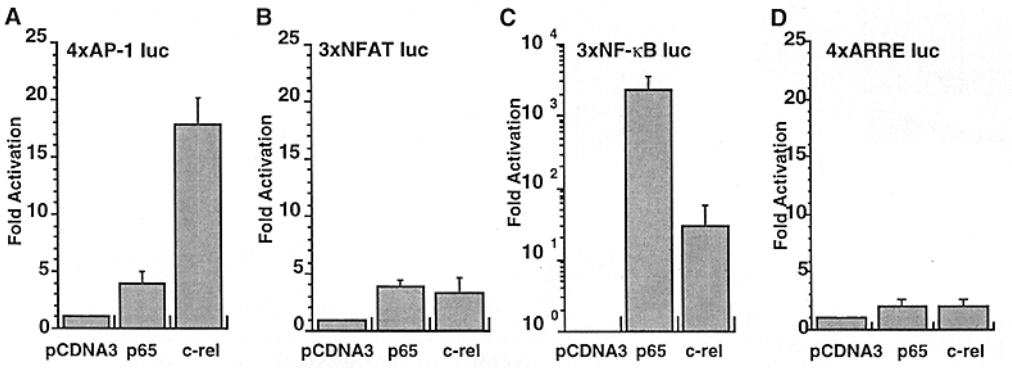

Figure 1. Jurkat TAg cells were transfected with 20 μg of either (*A*) 4×AP-1 luc, (*B*) 3×NF-AT luc, (*C*) 3×NF-κB luc, or (*D*) 4×ARRE luc and 40 μg of either control vector pCDNA3 or expression constructs for p65 or c-rel. After 2 d, 10^5 live cells (~1/50 of the total cells from each transfection) was examined for luciferase activity. The results represent the average of three independent transfections. Within each experiment, the fold activation was calculated for each construct relative to the luciferase activity of the construct transfected with pCDNA3 alone. Error bars reflect the standard error of the mean. Note that the scale is different in (*C*).

To generate KKK:

5′-TGAACCAACAAGAAGTAAAAGAAGCTATT-3′
3′-CCCATAAGCTACACACTTGGTTGTTCT-5′

Cell Culture, Transfections, and Stimulations. Jurkat TAg cells (provided by Dr. G. Crabtree) were grown in RPMI supplemented with 10% fetal calf serum. The total amount of DNA transfected within each experiment was kept constant by addition of the vector pCDNA3 where appropriate. 1×10^7 Jurkat TAg cells were transfected in a total volume of 0.4 ml in RPMI (without fetal calf serum) by electroporation using a BioRad Gene Pulser Electroporator set at 250 V, 960 μF. 2 d after transfection (40–48 h), the number of live cells within each sample were counted by Trypan blue exclusion. Luceriferase assays were performed in triplicate within each experiment using 1×10^5 live cells in a total volume of 90 μl. Typically, this represents 1/50 of the total live cells recovered after 2 d. Samples were stimulated, where appropriate, for 6 h with a 1:1,000 dilution of C305 ascites (anti-clonotypic Jurkat TCR) or a combination of 50 ng/ml PMA and 1 μM ionomycin (Calbiochem Novabiochem, San Diego, CA). All transfections were done on three separate occasions, and the results represent the average of three independent transfections, with the error bars representing the standard error from the mean.

Luciferase Assays. To the 90-μl cell sample (containing 1×10^5 live cells), 10 μl of cell harvest buffer containing 10% Triton X-100, 4 mM DTT, and 200 mM $NaPO_4$, pH 7.8, and 100 μl of assay buffer containing 200 mM $NaPO_4$, pH 7.8, 20 mM $MgCl_2$, and 10 mM ATP was added. Luciferase activity was measured after luciferin addition either manually or in a microplate luminometer.

Results

c-rel Overexpression Activates AP-1, but Not NF-AT or ARRE-1 Reporter Constructs. To determine whether c-Rel could regulate IL-2 gene expression by increasing the activity of transcription factors involved in IL-2 promoter regulation, c-Rel was overexpressed in transient transfection assays using Jurkat TAg cells in combination with reporter constructs containing NF-AT, ARRE-1 (Oct/OAP), AP-1, and NF-κB sites. The empty vector pCDNA3 was included as a negative control. The related NF-κB family member p65 was also included to determine whether c-Rel and p65 could differentially regulate these reporter constructs. As shown in Fig. 1 *A*, c-Rel dramatically enhanced AP-1 reporter activity, ~17-fold greater than vector alone. This upregulation was in the absence of any stimuli. In contrast, p65 overexpression had a milder effect on AP-1 reporter activity, enhancing it approximately fourfold. These results are consistent with previous reports, where p65 overexpression upregulated AP-1 reporter activity by approximately fivefold (30). Therefore, c-Rel was a more potent activator of AP-1 reporter activity than p65. However, p65 was a better activator of an NF-κB reporter (Fig. 1 *C*) with ~100-fold greater activity than c-Rel. In contrast with the dramatic effects of c-Rel overexpression on AP-1 reporter activity, only minimal effects on NF-AT and ARRE-1 reporter activity were observed (Fig. 1, *B* and *D*), although these reporters could be activated by a combination of PMA and ionomycin (data not shown). In addition, p65 overexpression did not affect NF-AT or ARRE-1 reporter activity. Therefore, c-Rel overexpression had the most dramatic effect on upregulating AP-1 activity, as compared with other transcription factors involved in regulating IL-2 promoter activity.

Stimulation and c-Rel Overexpression Cooperate in AP-1 Activation. To determine whether AP-1 reporter activation depended on the extent of c-Rel expression, we performed a dose response analysis. As shown in Fig. 2 *A*, activation of AP-1 activity increased proportionately with increasing amounts of transfected c-Rel. Transfections using increasing amounts of p65 did not lead to a dose-dependent effect on AP-1 activity. Since AP-1 activity is also increased in stimulated T cells, we examined the effects of c-Rel overexpression on TCR or PMA/ionomycin-mediated AP-1 activation. As shown in Fig. 2 *B* and 2 *C*, overexpression of c-Rel enhanced AP-1 activation after stimulation with either anti-TCR antibodies or with a combination of PMA and ionomycin. This effect appears to be additive, and titratable across c-Rel concentrations. Overexpression of p65 does

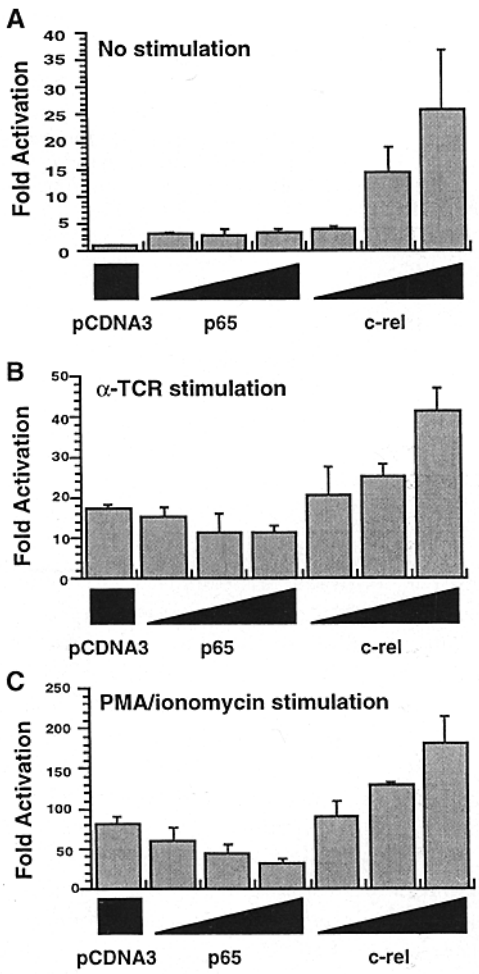

Figure 2. Jurkat TAg cells were transfected with 20 μg of 4×AP-1 luc and either 40 μg of control vector pCDNA3 or increasing amounts (5 μg, 10 μg, or 20 μg) of p65 or c-rel expression constructs. In all cases, pcDNA3 was added where appropriate to standardize the total amount of DNA transfected to 60 μg total per sample. After 2 d, 10^5 live cells (~1/50 of the total cells from each transfection) were left unstimulated, or stimulated with either 1:1,000 dilution of C305 anti-TCR ascites or 50 mg/ml PMA with 1 μM ionomycin. After 6 h, the cells were examined for luciferase activity. The results represent the average of three independent transfections. The fold activation was calculated relative to the luciferase activity of unstimulated cells transfected with pCDNA3 alone. Error bars reflect the standard error of the mean. Note that the scale is different in each graph.

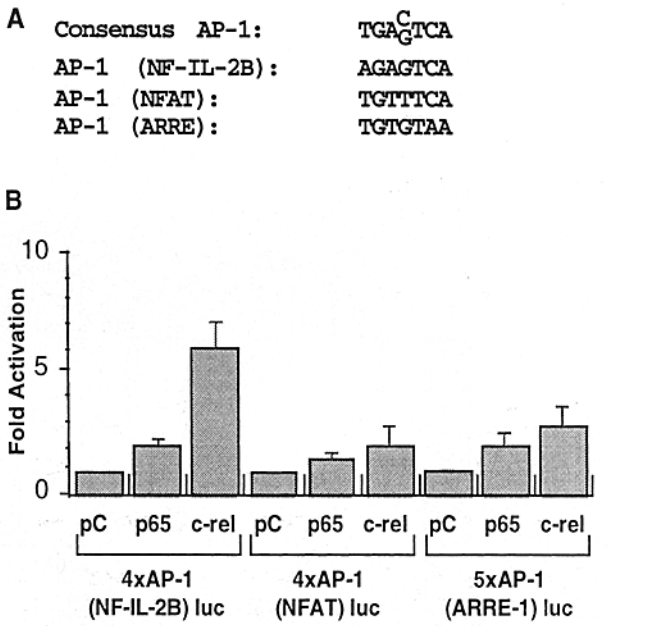

Figure 3. (*A*) The sequence of the AP-1 sites from the distal NF-AT site, the ARRE-1 site, and the NF-IL-2B site from the IL-2 promoter is compared with the consensus AP-1 sequence. (*B*) Jurkat TAg cells were transfected with 20 μg of either 4×AP-1(ARRE) luc, 5×AP-1(NF-AT) luc, or 4×AP-1(NF-IL-2B) luc and 40 μg of either control vector pCDNA3 or expression constructs for p65 or c-rel. After 2 d, 1×10^5 live cells (~1/50 of the total cells from each transfection) was examined for luciferase activity. The results represent the average of either two (4×AP-1[NF-IL-2B] luc) or three (4×AP-1[NF-AT] luc or 4×AP-1[ARRE] luc) independent transfections. Within each experiment, the fold activation was calculated for each construct relative to the luciferase activity of the construct transfected with pCDNA3 alone. Error bars reflect the standard error of the mean.

not increase AP-1 activity in response to either anti-TCR antibodies or with PMA/ionomycin. Therefore, c-Rel increases the amount of AP-1 activity in the presence or absence of additional stimuli such as via the TCR or pharmacologic agents.

Overexpression of c-Rel also Increases the Activity from the AP-1 Site of the IL-2 Promoter (NF-IL-2B). As described above, even though c-Rel appears to be a potent activator of AP-1, c-Rel had little effect on either NF-AT or ARRE reporter constructs (see Fig. 1). However, both the NF-AT site and the ARRE-1 site contain nonconsensus binding sites for AP-1, and AP-1 has been demonstrated to bind to these sites after T cell activation. Additionally, there is another nonconsensus AP-1 site in the IL-2 promoter, the NF-IL-2B site. To examine the effects of c-Rel overexpression on AP-1 sites from the IL-2 promoter specifically, reporter constructs containing only the AP-1 sites from either the distal NF-AT site, the ARRE-1 site, and the NF-IL-2B AP-1 site were created. A comparison of these AP-1 sites is shown in Fig. 3 *A*. As shown in Fig. 3 *B*, the activation of c-Rel on the nonconsensus NF-IL-2B AP-1 reporter was significant but less than observed with the consensus AP-1 reporter (see Fig. 1 *A*), 6-fold as compared with 17-fold, respectively. However, although c-Rel mediated activation on the nonconsensus NF-IL-2B AP-1 reporter is weaker than on a consensus AP-1 reporter, it is still greater than the effects of p65 overexpression on a consensus AP-1 reporter (see Fig. 1 *A*; 30). This suggests that one way c-Rel may regulate IL-2 gene expression is through the NF-IL-2B AP-1 site. p65 overexpression had minimal effects on NF-IL-2B AP-1 reporter activity. Only minor effects are observed by c-Rel or p65 overexpression on either of reporters constructed with the nonconsensus AP-1 sites from the distal NF-AT or ARRE-1 sites. It should be noted that these nonconsensus AP-1 elements may not function without the adjacent NF-AT or Octamer sites. Indeed, AP-1 is unable to bind to the distal NF-AT site in the absence of NF-AT protein (33). Therefore, these AP-1 sites from NF-AT and ARRE-1 may not function as independent

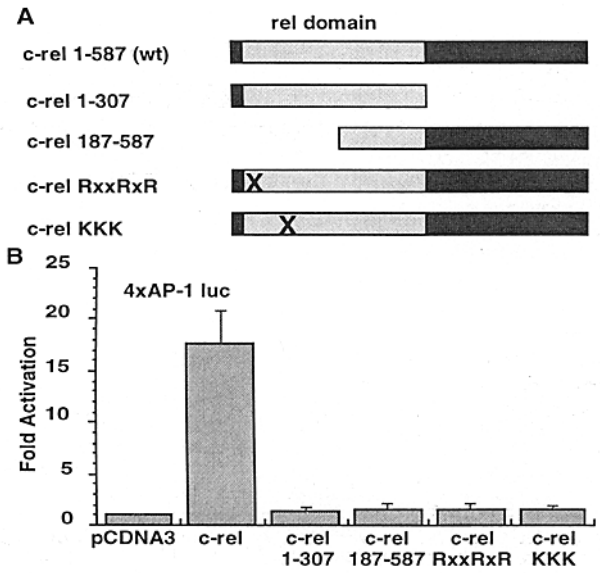

Figure 4. (*A*) Shown schematically are various deletion mutants and site-directed mutants of c-rel relative to wild-type. The lighter NH_2-terminal half of c-rel represents the rel domain, which is involved in DNA binding and dimerization. The darker COOH-terminal half of c-rel contains the transactivation domains. The X marks the placement of the mutated residues within c-rel. (*B*) Jurkat TAg cells were transfected with 20 μg of 4×AP-1 luc and 40 μg of either control vector pCDNA3 or different expression constructs for wild-type or mutant c-rel. After 2 d, 1×10^5 live cells (~1/50 of the total cells from each transfection) was examined for luciferase activity. The results represent the average of three independent transfections. Within each experiment, the fold activation was calculated for each construct relative to the luciferase activity of 4×AP-1 luc transfected with pCDNA3 alone. Error bars reflect the standard error of the mean.

AP-1 sites, which may reflect the lack of activation by either c-Rel or p65 overexpression. These results suggest one way through which c-Rel may regulate IL-2 gene transcription, through activation of the NF-IL-2B AP-1 site.

Both the DNA Binding Domain and the Transactivation Domains of c-Rel Are Required for AP-1 Activation. To determine which domains of c-Rel were responsible for upregulating AP-1 reporter activity, various c-Rel mutants were generated (shown schematically in Fig. 4 *A*). c-Rel 1–307 consists of only the Rel domain, which contains the DNA binding and dimerization sequences as well as the nuclear localization signal but lacks all previously described activation domains (31). c-Rel 187–587 has a deletion within the DNA binding domain of c-Rel, but contains the dimerization domain, nuclear translocation signal, and transactivation domains (31). c-Rel RxxRxR and KKK recreate specific DNA binding mutants that were originally described by Kumar et al. (32) in v-Rel. The homologous residues within the p50 *rel* homology domain were found in the crystal structure to be specific DNA contact residues (34), suggesting that these mutations eliminate DNA binding by altering direct interactions rather than by indirect structural perturbations. As shown in Fig. 4 *B*, elimination of either the DNA binding domain, by either deletion or specific mutation, or the transactivation domains abolished the ability of c-Rel to increase AP-1 activity. Therefore, both of these domains are critical for the activation of AP-1 by c-Rel.

Discussion

We have demonstrated that c-Rel substantially upregulates activity of an AP-1 reporter. This effect is not observed with either an NF-AT or ARRE-1 reporter. Moreover, c-Rel can activate an AP-1 reporter constructed from the NF-IL-2B site of the IL-2 promoter. The activation of AP-1 by c-Rel required both the DNA binding and transactivation domains of c-Rel. These data suggest that c-Rel is not increasing AP-1 reporter activity by functioning as a coactivator. If c-Rel functions as a coactivator, one would expect to be able to mutate the DNA binding domain of c-Rel and still increase AP-1 reporter activity when overexpressed. However, as shown in Fig. 4 *B*, two distinct site-directed mutants of c-Rel that specifically disrupted DNA binding did not upregulate activity from an AP-1 reporter. Additionally, we attempted to determine whether c-Rel could bind directly to AP-1 family members bound to an AP-1 oligonucleotide in gel mobility shift assays. Using a recombinant GST–c-*rel* fusion protein, we were unable to demonstrate a shift in the AP-1 complexes when GST–c-*rel* was added (data not shown). These data argue against, although not eliminate, the possibility that c-rel is functioning as a coactivator to upregulate AP-1 activity. It is also possible that c-rel mediates the AP-1 activation indirectly, by increasing expression of a cytokine or other soluble factor, which, in turn, upregulates AP-1 activity. However, mixing experiments argue against this idea. Specifically, Jurkat TAg cells, transfected either with 4×AP-1 luc or with a c-rel expression vector, were mixed together. Upregulation of AP-1 activity would have implicated a soluble factor in c-rel-mediated AP-1 activation. However, no activation of AP-1 was detected (data not shown). Therefore, it does not appear that c-rel mediates AP-1 activation by functioning as a coactivator, by binding directly to AP-1 sites, or by producing a cytokine or other soluble factor, which, in turn, feeds back to increase AP-1 activity.

In activated T cells, AP-1 activity is regulated by two mechanisms: levels of AP-1 family members (fos/jun) are transcriptionally upregulated and their relative activity is increased by phosphorylation (35). Since c-rel is a transcription factor, c-rel may upregulate AP-1 activity directly by increasing AP-1 protein levels through an increase in transcription of AP-1 family members. However, we were unable to detect either an increase in AP-1 levels using gel mobility shift assays or an increase in the protein levels of fos/jun family members by Western blot analyses from c-Rel overexpressing transiently transfected Jurkat TAg cells (data not shown). This may be due to limitations within our transient expression system. Since we are unable to detect AP-1 protein in transiently transfected cells, we also cannot examine an alternate possibility in which c-rel activates AP-1 through increased phosphorylation. c-rel and JNK1

have been reported to interact in vivo, although the functional significance of this interaction is not known (36). It is possible that c-rel could directly or indirectly activate JNK1 to influence AP-1 reporter activity. The elucidation of the mechanism by which c-rel increases AP-1 activity will depend on the development of a stable, inducible c-rel overexpression system, which is currently under investigation.

The critical role for c-rel in IL-2 gene expression was discovered using mice deficient for c-*rel*. Interestingly, this phenotype of mature T cells in the c-*rel*-deficient mouse, characterized by an inability to produce IL-2 and proliferate in response to stimuli, closely resembles what is observed in anergic T cell clones (reviewed in 37–38). The failure of anergic T cell clones to produce IL-2 is due to an inability to upregulate AP-1 activity after stimulation (39), demonstrating the essential role of AP-1 in IL-2 gene induction. Our results show that c-rel overexpression leads to increased AP-1 activity. Therefore, the failure of T cells from c-*rel* deficient mice to produce IL-2 may be due, at least in part, to a secondary decrease in AP-1 activation. The activation of AP-1 by c-rel provides one mechanism by which c-rel functions to regulate IL-2 gene expression.

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